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# Earthworms: a down-under delivery service for biocontrol agents of root disease

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Two common earthworms (*Aporrectodea trapezoides* and *A. rosea*) from cereal soils in southern Australia dispersed the biocontrol bacterium, *Pseudomonas corrugata*, through soil in the laboratory. Three experiments are reported. In the first *P. corrugata* survived passage through the intestine of both species of the earthworms. Worms in soil were fed fresh sheep dung containing $5.5 \times 10^8$ *P. corrugata* colony-forming units (cfu) per gram and produced casts containing $10^5$–$10^6$ *P. corrugata* cfu per gram. An average of $10^6$–$10^7$ *P. corrugata* cfu were recovered per worm of which 1% came from the homogenised worm after it had voided its gut. In the second experiment, the tunnelling and casting behaviour of both species was examined in 20 cm horizontal tubes of soil. Worms were applied at one end and within 16 days tunnels and casts were throughout the length of the tube. In the third experiment, worms and a mixture of sheep dung and *P. corrugata* were applied to one end of horizontal tubes of pasteurised soil. After 4 and 8 days, samples of cast, empty tunnel and unaffected soil were taken at 5, 10, 15 and 20 cm from where the dung and worms were applied. Both species of worm transported *P. corrugata* through the tube of soil. *A. trapezoides* moved the bacteria further and more quickly than did *A. rosea.* By day 8, bacteria were present 15–20 cm from the origin. No bacteria were recovered from the soil away from the point of introduction in control tubes with bacteria but no worms. Similar densities of *P. corrugata* were present in the worm casts and the soil associated with the walls of the tunnels ($10^3$–$10^6$ cfu per gram).

## 1. Introduction

The annual cost to the agricultural community of root pathogens of cereal crops has been estimated at $300m in Australia (A. D. Rovira pers. comm.), and worldwide this cost is many times greater. Root diseases are currently limiting world grain production, and so ineffective control of disease is a major factor constraining increased global food production.

Broadscale biological control of soilborne pathogens by introduced microorganisms has only recently been considered commercially feasible and several companies now have programs to develop biocontrol agents as commercial products (Weller 1988). Effective biological control of root pathogens of cereal crops and pastures often depends upon achieving a close association between the growing root and the protective agent so that the advances of the pathogen may be resisted. This requires both an effective biocontrol agent and its efficient dispersal through the rhizosphere, especially at the rhizoplane, the narrow zone that constitutes the interface between the plant root and the soil (Gammack et al. 1992).

While a variety of promising biocontrol microorganisms are now available (Weller 1988, Ryder et al. 1990, Gammack et al. 1992), the slowness of their unaided dispersal through the soil is a major factor limiting their effectiveness in controlling pathogens of growing roots. The soil macrofauna, especially earthworms, can act as vectors for soil microorganisms (Madsen & Alexander 1982, Opperman et al. 1987, Reddell & Spain 1991, Titi & Ulber 1991) and so provide a vehicle for dispersal of biocontrol agents through the root zone. However, there is a lack of quantitative data on the importance of these vectors in terms of distance moved and numbers of microbes carried (Gammack et al. 1992).

Earthworms are ideally suited to act as vectors of microbial biological control agents because they feed on organic matter which they redistribute through the root

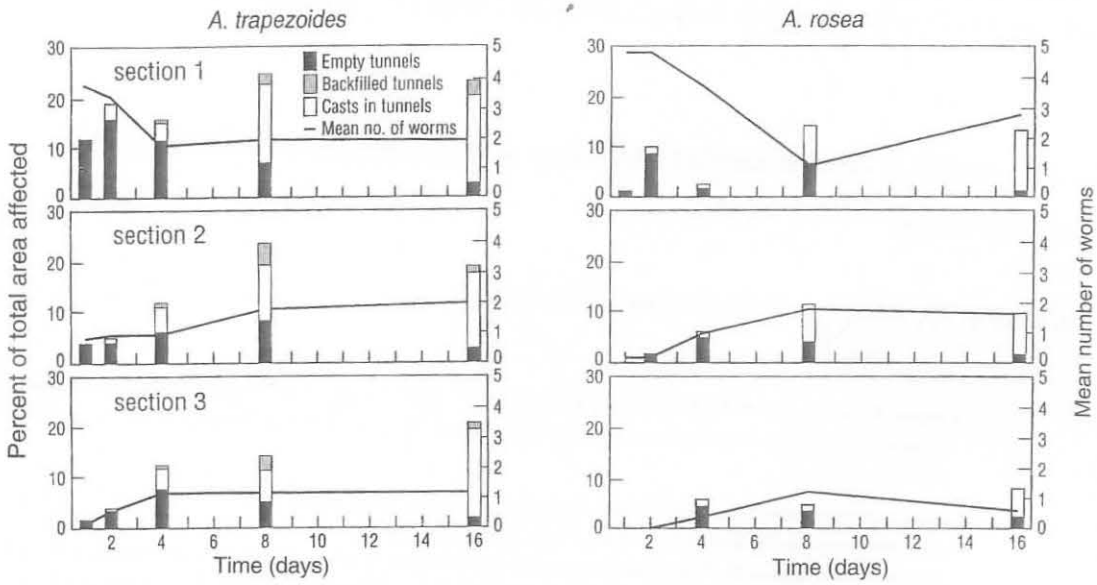


Fig. 1. The tunnelling and casting behaviour of *A. trapezoides* and *A. rosea* in tubes of soil.

zones of plants as excreta (casts) and tunnel linings (Lee 1985). Further, plant roots grow preferentially along planes of low resistance; e.g. down worm tunnels, and so tunnels lined with biocontrol agents could provide a niche in which plant roots are protected from pathogens in an otherwise hostile soil.

For earthworms to act as effective vectors of microorganisms, the microorganisms must either survive passage though the intestine of the worm or be transported on its outer surface, and must become lodged in situations into which plant roots are likely to grow. Here we show that two of the most common species of earthworms found in the cereal-growing soils of southern Australia are capable of dispersing substantial numbers of a biocontrol strain of *Pseudomonas corrugata* Roberts and Scarlett, over moderate distances through soil over a short period of time.

## 2. Materials and methods

Three sets of observations are reported. Two data sets concern a strain of the biocontrol bacterium, *P. corrugata* 2140 (Ryder & Rovira 1993), which was resistant to the antibiotic, Rifampicin.

In the first, the ability of *P. corrugata* to survive passage through the intestine of the earthworms *Aporrectodea trapezoides* (Duges) and *Aporrectodea rosea* (Savigny) is examined at 16°C. (*A. trapezoides* is a member of the *A. caliginosa* (Savigny) group of species but is quite distinct from *A. caliginosa* (unpublished data).) Field-reared adult earthworms of both species were collected and held for two days in pasteurised soil before being placed (in groups of about 20 individuals per species) in small plastic boxes containing about 1 kg of either a red-brown earth or a black clay soil. 85 grams of a mixture of fresh sheep dung and $4.7 \times 10^{10}$ *P. corrugata* were placed on the soil surface. The soil was pasteurised 2 days before the experiment began.

After 2 and 9 days the surface of the worms and their casts (excreta) were tested for the presence of Rifampicin-resistant *P. corrugata*. On both occasions ten individuals from each of the four boxes were removed, washed in three rinses of distilled water, confined individually in small flat plastic bags and held overnight at 16°C. The earthworms voided their gut content overnight and the cast material was collected the following morning. The casts produced by five worms of each species in each soil type were weighed, suspended in water and serially diluted and plated out on Nutrient Agar containing cycloheximide (75 mg/l) and rifampicin (100 mg/ml). In order to assess the number of bacteria remaining on the worm surface after washing the worms in water, the worms tested on day 9 were allowed to crawl for 5–10 seconds over the surface of plates of Nutrient Agar containing rifampicin (100 mg/l) before being placed in their bag.

The relative numbers of *P. corrugata*: i) in the recovered casts, ii) associated with the worm and iii) in the plastic bag after removing the casts and worm, were determined in another set of observations using procedures similar to those described above. Five boxes of each soil type were set up for each worm species (20 boxes) with *P. corrugata*-infested dung. After 9 days worms were removed from each box and held individually overnight in plastic bags. After 16 h the worms were inspected and worms with apparently empty intestines were selected for analysis. One worm from each box was examined: the casts and the worms were removed and the bag rinsed with

distilled water. The casts, homogenised worms and bag-washings were assessed separately by serial dilution for the presence of *P. corrugata*.

The presence of viable *P. corrugata* in the intestines of worms from the boxes was assessed by plating out worm gut. Worms were removed from the boxes, washed 3 times in distilled water then washed vigourously for 15–20 seconds in 70% ethanol to kill bacteria on the outside of the worms. Worms were rinsed again in distilled water and then wiped and rolled across plates of medium to test for the presence of bacteria on the outside of the worms. The gut of the worms was removed by dissection, weighed and homogenised, and the number of cfu of *P. corrugata* was determined.

The second set of observations concerns the rate of tunnelling and casting of both species of worms at 20°C. Groups of 5 earthworms were introduced to one end of a horizontal tube 21 cm long and 7 cm in diameter containing a red-brown earth at field capacity. After 1, 2, 4, 8 and 16 days tubes of soil were dismantled into 3 sections (each 7 cm long) and the cores of soil were extruded. There were three replicates for each species at each time interval. The amount of tunnelling and casting on the outer surface of each soil core was recorded by surrounding each core with transparent plastic and marking the area occupied by: i) empty tunnels, ii) trafficable tunnels containing casts and iii) tunnels backfilled with casts. The area ascribed to each category was estimated using a computerised video image analyser and expressed as a proportion of the total surface area. The number of worms in each section of each core was recorded.

In the third set of observations the capacity of both species of earthworms to disperse *P. corrugata* through cores of soil was examined at 20°C in the laboratory. A mixture of Rifampicin-resistant *P. corrugata* ($3.5 \times 10^{10}$) and fresh sheep dung was applied to one end of tubes of soil (similar to those described for the previous experiment) after 5 worms had been added to each tube. The worms and dung were confined by gauze held in place by a cap. After 4 and 8 days sets of tubes were disassembled and sampled for *P. corrugata* at 5, 10, 15 and 20 cm from where the dung was applied, provided that the worms had reached that sampling point. Samples of cast, empty tunnel and unaffected soil were taken at each sampling point and tested by serial dilution for the presence of *P. corrugata*. Control tubes without earthworms were also set up.

Table 1. The mean wet weight of worms (g) and worm casts (g/g worm), and numbers (mean cfu/g ± *SE*) of *P. corrugata* recovered from the surface (Ind. surf.) and casts (Ind. cast) of the earthworms *A. trapezoides* and *A. rosea*.

| Treatment | Worm | Cast | Ind. surf. | Ind. cast ($\log_{10}$ n) |
|---|---|---|---|---|
| *A. trapezoides* | | | | |
| Red-brown earth | | | | |
| 2 days | 1.4 | 0.090 | – | 6.0 ± 0.2 |
| 9 days | 1.3 | 0.088 | 540 ± 190 | 5.9 ± 0.4 |
| Black clay soil | | | | |
| 2 days | 1.3 | 0.058 | – | 6.2 ± 0.3 |
| 9 days | 1.3 | 0.059 | 620 ± 230 | 6.0 ± 0.3 |
| *A. rosea* | | | | |
| Red-brown earth | | | | |
| 2 days | 0.6 | 0.006 | – | 6.0 ± 0.2 |
| 9 days | 0.6 | 0.050 | 260 ± 110 | 5.4 ± 0.2 |
| Black clay soil | | | | |
| 2 days | 0.6 | 0.065 | – | 5.9 ± 0.3 |
| 9 days | 0.7 | 0.071 | 40 ± 14 | 4.9 ± 0.1 |

## 3. Results

Bacteria were present on the surface of the worms and in the casts of both species of worm and in both soil types (Table 1). Casts contained ca. $10^5$–$10^6$ *P. corrugata* per g of fresh cast and there were no significant effects of soil type or worm species on the density of bacteria in casts. The data from the partitioning experiment was highly variable and the total number of cfu per bag (containing one worm) ranged from $10^5$ to $10^8$ with an average of about $10^6$. In this experiment also there was no effect of soil type on the number of bacteria present in casts or on the total number associated with each worm and so the data for soil type were pooled to produce Table 2. Similar numbers were found in association with both *A. trapezoides* and *A. rosea* despite the difference in worm size (Table 2). About 39% of *P. corrugata* were recovered from the casts and a further 60% were present in washings from the bag: only 0.5–1.0% was associated with the worms. All worms had voided their gut contents before the tests were carried out.

Washing worms in 70% alcohol for 10–15 seconds resulted in very few or no bacteria being recovered from the outer surfaces of some worms but other worms produced up to several hundred cfu. Because we wished to avoid the possibility of contamination during dissection of the worm gut by bacteria from the outer surface of the worm, we have considered only those worms from which fewer than 40 viable colonies were recovered from the outer surface. In these individuals considerable numbers of *P. corrugata* were recovered from the gut (a mean of $10^4$–$10^5$ per g of wet gut; $n = 10$ for *A. trapezoides* and n = 8 for *A. rosea*), indicating unequivocally that viable bacteria were present in guts of both worm species. These counts (per g gut) were lower than those of casts (per g cast).

Table 2. The numbers (× 1000 cfu, mean ± *SE*) of *P. corrugata* recovered from the empty worm, its casts and the plastic bag in which it was maintained overnight in order to produce its casts. Ten individual *A. trapezoides* and *A. rosea* were tested.

| | Casts | Worm | Bag | Total |
|---|---|---|---|---|
| *A. trapezoides* | 396 ± 188 | 7 ± 1.9 | 605 ± 184 | 1009 ± 355 |
| *A. rosea* | 326 ± 142 | 14 ± 11 | 581 ± 140 | 921 ± 261 |

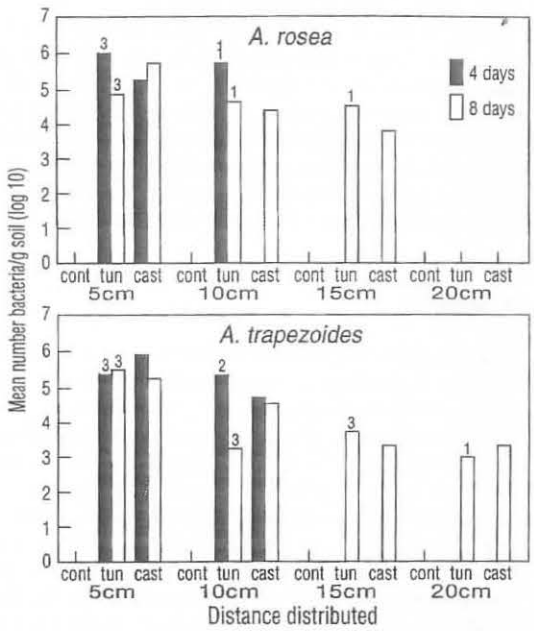
A. rosea
4 days
8 days
A. trapezoides
Mean number bacteria/g soil (log 10)
cont tun cast cont tun cast cont tun cast cont tun cast
5cm 10cm 15cm 20cm
Distance distributed

Fig. 2. Redistribution of *P. corrugata* through cores of soil by *A. trapezoides* and *A. rosea* at 20°C. After 4 and 8 days samples of worm casts, tunnel walls (tun) and control soil unaffected by earthworms (cont) were collected and the numbers of cfu per g of soil assessed. Numbers above each column indicate the number (out of 3) of replicates in which earthworms had reached that section of the tube.

The tunnelling and casting behaviour of both worm species is shown in Fig. 1. The larger worm, *A. trapezoides*, tunnelled further and faster and affected more of the soil than did *A. rosea*. Initially, worms tunnelled largely in the soil adjacent to the point at which they were introduced to the core, but by days 4 and 8 worms were evenly dispersed throughout the three sections of the cores. By day 8, 10–25% of the surface area had been affected by *A. trapezoides* and 5–15% by the smaller worm, *A. rosea*. Of this affected area, most was tunnel containing some casts but there was a small proportion of tunnel completely backfilled with casts.

Both species of worm transported *P. corrugata* through the tube of soil (Fig. 2). After 4 days both species of earthworm had moved bacteria 5–10 cm from the origin. By day 8 *A. trapezoides* had moved the bacteria 20 cm from the origin. No bacteria were recovered from the soil away from the point of introduction in control tubes with bacteria but no worms. *P. corrugata* was present in both the casts and in the soil associated with the tunnels (with no obvious casts present). Similar densities of bacteria were present in the worm casts and in the soil associated with the walls of the tunnels. The density of bacteria in both the casts and tunnel walls decreased as the distance from the source of infestation (the dung) increased (Fig 2). Similar densities of bacteria were present in casts made by both species of earthworm.

## 4. Discussion

The importance of soil animals for microbial dispersal in soil is generally assumed but has rarely been quantitatively studied (Weller 1988, Gammack et al. 1992). Our experiments demonstrate that both *A. trapezoides* and *A. rosea* have the capacity to ingest a mixture of the biocontrol agent, *P. corrugata* 2140, and organic matter (e.g. sheep dung) and quickly to disperse the bacteria horizontally through the soil. The bacteria are carried both on the surface of the worm and in its intestine. Large numbers (about $10^6$ per g) were recovered from the cast after the worm had been separated from the source of *P. corrugata* for some time and only a small proportion of the bacteria were associated with the worm once it had voided its gut contents. Whether any of the bacteria are digested during passage through the gut is not known, but it is clear that substantial numbers can be present in the casts 16 h after removal from a source of bacteria. It may be that the bacteria multiply in worm casts and dermal secretions from the worms, but this is not known and needs to be examined.

Bacteria can both proliferate and be digested during passage through the guts of earthworms (Lee & Foster 1991, Titi & Ulber 1991). Lee & Foster (1991) recognised the empty remnant cell walls of bacteria and clay cutans or 'ghosts' of bacteria in the casts of earthworms. Their further observation that casts also contained living bacteria in protected sites within soil microaggregates or as clay-coated microcolonies suggested that the clay may have protected bacteria from digestion. In our experiments the numbers of bacteria recovered were not affected by soil type, the clay contents of which were 11% (red-brown earth) and 30% (black soil). However, it is possible that the clay contents of both the soils we used were both above a threshold level for protecting bacteria from digestion. Alternatively the rich diet of the worms (sheep dung) may have protected the bacteria from digestion. A different result may have arisen if our experiments had been conducted using sand and a worm food of lower nutritional value.

It was of special interest to find that *P. corrugata* was found both in the casts and in the worm tunnels made by both *A. trapezoides* and *A. rosea*. Unpublished data concerning *A. trapezoides* which were fed dyed sheep dung have shown that worm tunnels become lined with dye, indicating that in some way dung materials accumulate on tunnel walls. These observations provide clear evidence that endogeic species line their tunnels with organic matter. The anecic species, *Lumbricus terrestris*, which lives in deep permanent tunnels, has long been known to line the walls of its tunnels with organic matter (Lee 1985).

Unpublished experiments by the authors comparing the behaviour of *A. trapezoides* in vertical and horizontal tubes of soil indicate similar levels of tunnelling and cast-

ing activity in all directions around a buried source of sheep dung. These experiments have been conducted using buried worm food, but it seems likely that similar effects could be achieved with surface-applied worm food. Certainly a number of species have been shown to move surface-applied organic matter down through the soil profile (Lee 1985).

Madsen & Alexander (1982) showed that the earthworm *Lumbricus rubellus* (Hoffmeister) increased the rate of dispersal of surface-applied *Pseudomonas putida* (Trevisan) into soil, but only small numbers were recovered at depths of up to 10 cm. This may reflect the fact that *L. rubellus* is largely a surface-active worm. In contrast, Opperman et al. (1987) showed that the activity of *Eisenia foetida* was associated with the dispersal of presumptive coliform bacteria to depths of 7.5 to 17.5 cm in sandy soil after 4 days. Our unpublished data suggest that the tunnelling and casting behaviour of *A. trapezoides* and *A. rosea* are likely to be concentrated in the root zone of field crops.

The slow rate of unaided dispersal of soil-borne biocontrol microorganims has been recognised as an important impediment to their effective use and Weller (1988) indicated the need for further research on the formulation and delivery of such organisms. Our observations raise the possibility that a pelletted formulation of organic worm food mixed with bacterial and/or fungal biocontrol agents could be used to achieve an extensive dispersal of biocontrol agents through the root zone of plants at risk to root diseases (Weller 1988, Gammack et al. 1992). Such pellets could be either applied to the soil surface or buried with the seed during sowing where they would be fed upon by natural worm populations and dispersed through the rhizosphere, providing nutrient-rich protected channels for root growth in a potentially hostile soil. The development of a system for delivering and dispersing effective agents for the biological control of cereal root diseases throughout the rhizosphere at the time of root proliferation would fill one major deficiency in our current ability to use biocontrol agents effectively for the control of root diseases.

The species *A. trapezoides* and *A. rosea* were selected for study because they are commonly encountered in the cereal-growing regions of southern and eastern Australia (Barley 1959, McCredie & Parker 1991, Baker et al. 1992). These species can be present at densities of hundreds per square metre, although such densities are commonly found only where conservation farming practices are followed (e.g. direct drilling of crops, stubble retention). At such densities it seems likely that worm-food pellets containing biocontrol agents would be quickly discovered by the resident worm populations with the result that the biocontrol agents would become dispersed through the root zone. Whether such processes could lead to effective biocontrol of root diseases of broad-acre cereal crops needs to be evaluated.

We also recognise the potential of this process for inoculating soils with *Rhizobium*, Amycorrhizal fungi, spores of biocontrol fungi and other beneficial ameliorants such as lime and trace elements.

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